

Estrogen-Induced Abnormal Accumulation of Fat Cells in the Rat Penis and Associated Loss of Fertility Depends upon Estrogen Exposure during Critical Period of Penile Development

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We previously reported that diethylstilbestrol (DES) or estradiol valerate (EV) exposure at a dose of 0.10–0.12 mg/kg, or higher, per day, on alternate days, from postnatal days 2–12, resulted in abnormal penis development and infertility (H. O. Goyal *et al.*, 2005, *J. Androl.* 26, 32–43). The objective of this study was to identify a critical developmental period(s) during which EV exposure results in the observed penile abnormalities. Male pups received EV at a dose of 0.10–0.12 mg/kg on postnatal day(s) 1, 1–3, 4–6, 1–6, 7–12, 13–18, 19–24, or 25–30. Fertility was tested at 102–115 days of age and tissues were examined at 117–137 days. Both penile morphology and fertility were unaltered in rats treated with EV after 12 days of age. Conversely, except in rats treated on postnatal day 1 only, none of the males treated prior to 12 days of age sired pups, and all had abnormal penises, including varying degrees of abnormal accumulation of fat cells and loss of cavernous spaces and smooth muscle cells in the corpora cavernosa penis, which were maximal in the 1–6-day group. Also, the preputial sheath was partially released or its release was delayed, and the weight of the bulbospongiosus muscle was significantly reduced. Plasma testosterone (T) in the 1–6- and 4–6-day groups and intratesticular T in the 4–6-day group were significantly lower. The testosterone surge, characteristic of controls in the first week of life, was suppressed in the 1–3-day group. Estrogen receptor alpha mRNA expression was enhanced in the body of the penis in the 1–3-day group, but not in the 13–18-day group. Hence, EV exposure prior to 12 days of age (as short as 1–3 days postnatal), but not after 12 days of age, results in long-term abnormal penile morphology, characterized by abnormal accumulation of fat cells in the corpora cavernosa penis and, consequently, loss of fertility.

Key Words: estrogen; DES; penis; development; toxicology.

Male reproductive disorders account for at least 40% of infertility cases in humans. Although causes of infertility in most men are multi-factorial, links have been made between

environmental estrogen exposure and lower sperm counts in men (Fisher, 2004; Jouannet *et al.*, 2001, reviews) and higher incidences of reproductive abnormalities in men and wildlife (Toppari *et al.*, 1996, review). Male offspring of women exposed to diethylstilbestrol (DES) during pregnancy have higher incidences of retention of testes, epididymal cyst, hypospadias, and small phallus (Gill *et al.*, 1979; Shawn, 2000, reviews). Laboratory animals exposed neonatally or prenatally to inappropriate amounts of estrogenic compounds exhibit reproductive abnormalities, including microphallus and hypospadias (McLachlan *et al.*, 1975; Toppari *et al.*, 1996; Newbold, 2001). Alligators from Lake Apopka (FL) contaminated with industrial effluents containing estrogenic chemicals, but not those from the control lake Woodruff, have smaller phalluses (Guillette *et al.*, 1994, 1996). Thus, exposure to estrogen or related xenobiotics during critical developmental periods can have lasting and often negative consequences for male reproductive health and fertility later in life.

Permanent penile dysfunction, including loss of fertility, can be induced in adult rats treated neonatally, but not at adulthood, with estrogens (Goyal *et al.*, 2004a). In this rat model of penile dysfunction, we reported that DES exposure at a dose of 10 µg per rat (approximately 1 mg/kg), per day, on alternate days, from 2–12 postnatal days, resulted in infertility in 100% of the treated males; and the loss of fertility was associated with abnormal accumulation of fat cells and loss of cavernous spaces and associated smooth muscle cells in the corpora cavernosa penis. Subsequently, in a dose-dependent study and using the above treatment schedule, we reported that neonatal exposure to DES or estradiol valerate (EV) at a dose of 0.10–0.12 mg/kg, or higher, resulted in similar histopathological abnormalities in the penis, as well as loss of fertility, in 100% of the treated rats, although a lower dose of DES (0.01 mg/kg) also caused infertility, but at a lower percentage (Goyal *et al.*, 2005).

Based upon observations from other studies (Murakami *et al.*, 1986, 1987) that both cavernous spaces and smooth muscle cells were absent in the rat penis at birth and started to differentiate past 5–7 days of age, we hypothesized that the structural changes that we observed in the corpora cavernosa

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penis probably resulted from estrogenic effects limited to the early period of penile development. Hence, the objective of this study was to determine if there is a critical developmental period(s) during which estrogen exposure results in the above reported penile abnormalities, including loss of fertility.

MATERIALS AND METHODS

Animals and treatments. Neonatal and/or adult Sprague-Dawley male and female rats (Harlan Sprague Dawley, Indianapolis, IN) were maintained at 22–23°C ambient temperature, 55–60% relative humidity, and 12L:12D cycle, and had free access to food (Rodent Chow 5001; Purina Mills, St. Louis, MO) and water for 24 h. Animals were handled in accordance with the guidelines stipulated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington, DC, 1996). All animal procedures were approved by the Institutional Animal Care and Use Committee at Tuskegee University.

Timed-pregnant female rats were housed individually. Within 24 h of delivery, 5–8 male pups from different litters (one pup from each litter, in order to avoid litter effect) were randomly assigned to each group, placed in separate cages, and the litter size per group was adjusted to eight pups with the remaining number of females. Pups received sc injections of 25 µl of olive oil containing EV (Sigma, St. Louis, MO) at a dose of 0.10–0.12 mg/kg per pup, each day, on postnatal day(s) 1, 1–3, 4–6, 1–6, 7–12, 13–18, 19–24, or 25–30 (dose was adjusted based on the weight of pups; postnatal day 1 is considered within 24 h of birth; and these groups will be referred in the text as 1-day group, 1–3-day group, so on). Controls received oil only for postnatal days 1–6. The above dose of EV was selected based on our previous study, where it caused abnormal penile morphology and infertility in 100% of the adult male rats treated with DES or EV on alternate days from 2–12 postnatal days (Goyal *et al.*, 2005). Animals were weaned at 22 days of age and regularly observed for development until tested for fertility at 102–115 days of age, and tissues were collected at 117–137 days of age.

Descent of testes. Testicular descent was observed every other day from 22 to 38 postnatal days and thereafter every third or fourth day (every Friday and Tuesday of the week). Testes were characterized as fully descended when they were palpated in the scrotum while holding the animal in a supine position. The formation of a scrotal bulge usually marked the descent in most animals.

Release of preputial sheath. The preputial sheath was examined every third or fourth day, from 38 to 101 days of age, one day prior to the fertility trial. While holding the animal in a supine position, the prepuce was gently pushed proximally (toward the abdominal wall) and was characterized as fully released when it completely retracted from the glans penis up to its transition with the body of the penis.

Body weight and organ weights. All animals were weighed and terminated at 117–137 days of age. The testis caput and corpus of the epididymis, cauda of the epididymis, and seminal vesicle (including coagulating gland) of both sides were weighed. Testes and epididymides were trimmed of fat prior to recording their weights. After weighing, testis and epididymis of the left side were frozen at –20°C until thawed for sperm counts. In addition, the caudal epididymal fat pad, located between the cauda epididymis and the distal extremity of the testis, was removed and weighed. The reason for including the caudal epididymal fat pad weight as one of the parameters was that, in our dose-dependent study (Goyal *et al.*, 2005), we found it to be as sensitive as, if not more than, the seminal vesicle to neonatal estrogen exposure.

Penis and penile skeletal muscles. The penis was measured for length, diameter, and weight, and was processed as described previously (Goyal *et al.*, 2005). Briefly, the penis was exposed up to the ischial arch, and its stretched length was measured from the tip of the glans penis to the mid point of the

ischial arch, and the diameter was measured from the middle of the body of the penis. After removing the free loose connective tissue, the entire penis was weighed. Three to five mm long sections from the middle of the body were processed for histopathology and histochemistry ($n = 4$ per group, except 3 each for the 1–3-, 1–6-, and 7–12-day groups). For histopathology, tissues were fixed in formaldehyde, and 5 µm-thick paraffin sections were stained with hematoxylin and eosin (H&E). For histochemical demonstration of fat, formaldehyde-fixed tissues were en block stained for 8 h with 1% osmium tetroxide dissolved in 2.5% potassium dichromate solution, and then processed for paraffin embedding.

Undeparaffinized sections from the body of the penis in each animal were quantified for fat cells with a Leica M8 stereomicroscope (Vashaw Scientific, Inc., Norcross, GA). Digital images of the corpora cavernosa were captured using a Kodak MDS 290 digital camera (Eastman Kodak Company, Rochester, NY) and a Macintosh G4 computer, processed to optimize details using Adobe Photoshop 7.0, and stored as psd files. The corpora cavernosa, including its capsule (tunica albuginea), were delineated using the “lasso” tool of Photoshop; and the area was determined using the “Measure Features – Measure Regions” Photoshop plug-in of Fovea Pro v.3 (Reindeer Graphics, Inc., Asheville, NC). The digital images were then copied and subjected to “thresholding” Photoshop plug-in of Fovea Pro v.3. After thresholding, entities outside the corpora cavernosa and nonfat cell entities within the corpora cavernosa were removed using the “pencil” tool of Photoshop. In addition, fat cells contacting each other or the edge of the field were separated by 2 pixels using the same tool. The number and area of fat cells were determined using the “Measure Features – Measure All Features” Photoshop plug-in of Fovea Pro v.3. Data were recorded as simple text, transferred to Microsoft Office EXCEL, and subjected to statistical analysis.

Adjacent serial sections stained with H&E were used for examination of histopathological details. Digital images were captured, processed, and stored as described above. In addition to histopathological and histochemical examination of the penis, the os penis, characteristic of the rodent glans penis, was radiographed as described previously (Goyal *et al.*, 2005); and radiographs were digitally scanned. The bulbospongiosus and levator ani muscles were isolated and weighed as described previously (Goyal *et al.*, 2005). Montage figures were assembled using Adobe Photoshop 7.0.

Plasma and intra-testicular testosterone. For plasma testosterone, one blood sample was collected from the heart of each animal just prior to necropsy; and for intratesticular testosterone, a part of the right testis was collected from each animal at the time of necropsy. Both plasma and testicular parenchyma were frozen at –20°C until assayed. In addition, in a separate experiment, one or both testes from rats treated with EV or oil (control) for 1–3 postnatal days were frozen on postnatal day 5, 8, 12, and 17 ($n = 2$ for each age group). The objective of this experiment was to find out whether EV treatment results in suppressing the neonatal intratesticular testosterone surge that is typical for rodents from late gestation (18.5–19.5 days) to the first week of life (El-Gehani *et al.*, 1998; Ward and Weisz, 1984). The reason for treating pups for 1–3 postnatal days was that, among various treatment groups, this was the earliest and the shortest developmental period in which estrogen exposure resulted in abnormal penis and loss of fertility (see results below). Testes were processed in accordance with the protocol described by Park *et al.* (2002). Briefly, approximately 100 mg of testicular tissue (6–15 mg in the case of 5–17-day-old testes) was homogenized in PBS. Eight volumes of ethyl ester were added to the homogenate and vortexed vigorously. The aqueous phase was snap-frozen, and the organic supernatant was transferred to a secondary tube and air-dried. Just prior to assay, samples were re-suspended in PBS. Testosterone in plasma and testicular tissue was measured using a COAT-A-COUNT testosterone radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) according to manufacturer’s protocol. The sensitivity of the assay was 0.2 ng/ml. All samples were quantified in a single assay, and the intra-assay coefficient of variation was 7%.

Daily sperm production, epididymal sperm numbers, and sperm morphology. The daily sperm production and epididymal sperm numbers, a measure of efficiency of spermatogenesis, and sperm morphology, a measure of normal

spermatogenesis, were compared between controls and the 1–3- and 13–18-day groups. The reason for studying sperm-related parameters was to find out whether the loss of fertility resulting from neonatal estrogen exposure was associated with altered sperm functions. The reason for studying these parameters in the 1–3- and 13–18-day groups was that they were the youngest developmental groups, which exhibited penile deformities and loss of fertility, or the lack thereof, respectively, as a result of neonatal EV exposure (see results below). After thawing the testis, the capsule was detached and weighed. The testicular parenchyma was homogenized in 50 ml PBS, as described previously from our laboratory (Goyal *et al.*, 2001). The homogenate was filtered through metal sieve to remove connective tissue, and the filtrate was used to count the number of homogenization-resistant spermatids/sperm in each sample in duplicate using a hemocytometer. Daily sperm production was calculated by dividing the total number of spermatids or sperm per gram of testicular parenchyma (testis weight minus weight of the capsule) by 6.1 days, the duration of step 19 spermatids in the seminiferous epithelial cycle (Robb *et al.*, 1978). The total number of homogenization-resistant sperm in the head and body of the epididymis and the tail of the epididymis was determined as described for the testis.

For sperm morphology, sperm were collected from the right tail of the epididymis, near its junction with the ductus deferens (Slott *et al.*, 1991), in 2 ml of PBS containing 10 μ l of formalin (37% formaldehyde). A 10- μ l drop of the diluted sample was placed on a glass slide, coverslipped, and examined with a phase contrast microscope (400X total magnification). Two hundred sperm from each animal were evaluated and classified as normal, head defect, middle piece defect, principal piece defect, proximal droplet, distal droplet, and detached head (Goyal *et al.*, 2001). It should be noted that sperm motility parameters were not studied.

Estrogen receptor (ER) α mRNA expression. ER α mRNA expression was determined in the body of the adult penis in control rats and the 1–3- and 13–18-day groups ($n = 4$, each group). The reason for including this parameter in the present study was to test our previous hypothesis that estrogen-induced penile abnormalities are associated with enhanced expression of ER α (Goyal *et al.*, 2004b). Reasons for making this determination in the 1–3- and 13–18-day groups were the same as explained above for evaluating sperm-related parameters.

Total RNA was isolated from the body of the penis and efferent ductules (positive control) using TRIZOL reagent (Invitrogen-Life Technologies Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentration of RNA was estimated at 260 nm using UV spectrophotometry; and the quality of RNA preparations was evaluated by running samples on 2% denaturing agarose gel stained with ethidium bromide, as described previously from our laboratory (Mansour *et al.*, 2001).

The RT-PCR analysis was performed as described previously from our laboratory (Mansour *et al.*, 2001) and by using the published primers for ER α (Hess *et al.*, 1997) and the positive control primers for amplification of a conserved, constitutively expressed mRNA (ribosomal unit protein, *Rig/S15*). Briefly, two micrograms of total RNA from the body of the penis and efferent ductules were reverse transcribed with 100 units of Moloney murine leukemia virus (M-MLV) RT following instructions for RETROscript kit (Ambion Inc., Austin, TX). An aliquot of 3 μ l of RT product was used for PCR, which was performed on a Robocycler (Stratagene, La Jolla, CA) in a 50 μ l reaction volume containing 1X *Tag* DNA reaction buffer, 1.5 mM MgCl₂, 0.125 mM of each dNTP, 0.5 μ M of each primer, and 1 unit of Super *Tag* DNA polymerase (Ambion, Austin, TX). The PCR conditions included: initial cycle at 95°C for 2 min; 30 cycles each at 94°C for 45 s (denaturing), 53°C for 45 s (annealing), and 72°C for 1.5 min (extension), with a final extension step at 72°C for 7 min. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining and UV illumination. The optical density of each PCR product was normalized to that of *Rig/S15* within the animal, and the relative intensity of bands was quantified by optical density analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Fertility. Three to five 102–115-day-old male rats from each group were transferred to mating cages floored with a mesh grid and cohoused with

untreated, 70-day-old females (1:1) for 12 days (note, females were not checked for cyclicity, prior to cohabitation). Cages were checked twice daily for the presence of copulatory plugs. The plug-positive females were separated and evaluated for the presence of sperm in vaginal washings. These females were killed on the fifteenth day of pregnancy, and those without plugs were killed on the fifteenth day after the end of cohabitation. The uterus was removed and examined for the number of implantation sites and live fetuses. In addition, both ovaries were removed and the number of corpora lutea was counted (Goyal *et al.*, 2004a).

Statistics. Statistical analyses were performed using Sigma Stat statistical software (Jandel Scientific, Chicago, IL). Analysis of variance was performed on all parameters. Treatment groups with means significantly different ($p < 0.05$) from controls were identified using Dunnett's test. When data were not distributed normally, or heterogeneity of variance was identified, analyses were performed on transformed data or ranked data.

RESULTS

Body Weight

The mean body weight at 117–137 days of age was similar between controls and treated rats, regardless whether neonatal pups were exposed to EV on postnatal day(s) 1, 1–3, 4–6, 1–6, 7–12, 13–18, 19–24, or 25–30 (Table 1). However, two animals each in the 1–6- and 7–12-day groups died between 80 and 97 days of age.

Organ Weight

Testis, epididymis, and seminal vesicle. The absolute weight of the testis, head and body of the epididymis, tail of the epididymis, and seminal vesicle did not significantly ($p < 0.05$) differ between controls and rats treated after 12 days of age, except for the weight of the seminal vesicle, which was significantly higher in the 13–18- and 25–30-day groups (Table 1). Conversely, significant weight reductions were observed for the testis and head of the epididymis in the 1–6-day group; for the tail of the epididymis in the 1–3-, 1–6-, and 7–12-day groups; and for the seminal vesicle in the 1–3-, 4–6-, and 1–6-day groups. Generally, among treatment groups, the maximal weight reduction for all organs was found in the 1–6-day group and, among organs, the maximal weight reduction was found in the seminal vesicle (almost 90% in the 1–6-day group). The relative weights (weight of the organ per 100 g body weight) showed essentially similar differences.

Caudal epididymal fat pad. The weight of the caudal epididymal fat pad was unaltered in animals treated after 12 days of age, but was significantly ($p < 0.05$) decreased in all groups treated prior to 12 days of age, with reductions ranging from 80% in the 1–6-day group to more than 20% in the remaining groups (Fig. 1).

Descent of Testes

Testes descended at 23 to 25 days of age in controls and the 1- and 25–30-day groups, but the descent was delayed from 1 to

TABLE 1
Effects of Neonatal Estradiol Valerate (EV) Exposure

	Body	Testis	H/B EP	Tail EP	SV
Control (5)	435 ± 8	3.82 ± 0.05	0.68 ± 0.01	0.50 ± 0.01	1.49 ± 0.05
PD 1 (5)	424 ± 5	3.76 ± 0.08	0.74 ± 0.02	0.52 ± 0.01	1.51 ± 0.05
PD 1-3 (8)	434 ± 7	2.94 ± 0.15	0.59 ± 0.03	0.41 ± 0.02*	0.73 ± 0.11*
PD 1-6 (3)	429 ± 24	1.86 ± 0.13*	0.34 ± 0.04*	0.26 ± 0.01*	0.19 ± 0.06*
PD 4-6 (5)	407 ± 6	2.99 ± 0.06	0.58 ± 0.01	0.42 ± 0.01	1.11 ± 0.05*
PD 7-12 (3)	425 ± 9	2.81 ± 1.07	0.54 ± 0.06	0.34 ± 0.04*	1.11 ± 0.07
PD 13-18 (5)	468 ± 11	3.87 ± 0.04	0.72 ± 0.04	0.54 ± 0.01	1.84 ± 0.11*
PD 19-24 (5)	401 ± 5	3.78 ± 0.03	0.72 ± 0.01	0.49 ± 0.01	1.67 ± 0.04
PD 25-30 (5)	413 ± 9	3.32 ± 0.45	0.63 ± 0.08	0.42 ± 0.05	1.85 ± 0.09*

Note. Effect of neonatal estradiol valerate (EV) exposure for various postnatal days (PD) on body weight (g) and paired absolute weight (g) of the testis, head and body of the epididymis (H/B EP), tail of the epididymis (Tail EP), and seminal vesicle (SV) in rats at 117-137 days of age. Data are expressed as mean ± SEM.

*Significantly different from controls ($p < 0.05$). Relative weights showed essentially similar differences at ($p < 0.05$) and thus were not included.

15 days, depending upon the neonatal period and/or the length of the treatment (Table 2).

Release of Preputial Sheath

The preputial sheath was completely separate from the glans penis at 47-50 days of age in controls and the 1-day group, except 1 of 5 animals in the 1-day group in which it separated at 87 days of age. Conversely, the separation occurred late by 5 to 12 days in the 4-6-, 13-18-, 19-24-, and 25-30-day groups. The preputial sheath was still partially attached ventrally at the time of sacrifice (117-137 days of age) in the 1-3-, 1-6-, and 7-12-day groups, except one animal in the 1-3-day group in

which it occurred at 55 days of age ($n = 5$ for each group, except 8 for the 1-3-day group, and 3 each for the 1-6- and 7-12-day groups).

Penile Measurements

The mean measurements for weight, length, and diameter of the penis were not significantly ($p < 0.05$) different between controls and rats treated after 12 days of age (Fig. 2), as well as in the 4-6- and 1-day groups, except one of five animals in the latter group that had a markedly smaller and lighter penis (34 mm in length and 174 mg in weight vs. 38-41 mm and 251-307 mg in other four animals). Conversely, all three parameters were significantly lower in the 1-3- and 1-6-day groups, except one of eight rats in the 1-3-day group. In the latter animal, the penis was as long as in controls (41 mm), but its weight was markedly lower than in controls (236 vs. 267-301), but still higher than in other seven group-mates (130-188 mg). The weight and diameter of the penis were significantly lower in the 7-12-day

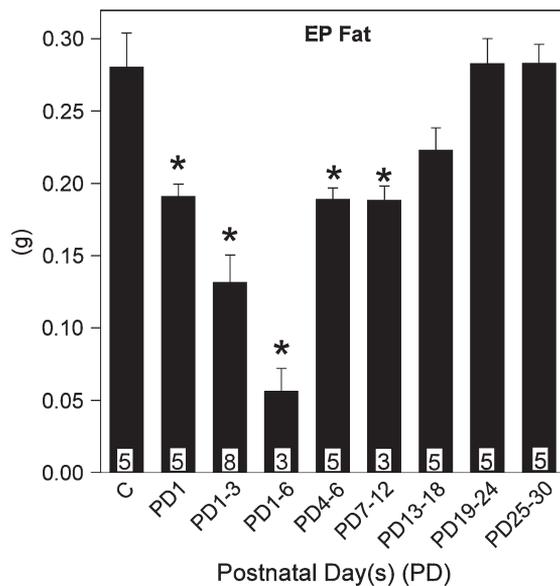


FIG. 1. Effect of neonatal estradiol valerate exposure for various postnatal days of age on the caudal epididymal (EP) fat pad in rats at 117-137 days of age. Data are expressed as mean ± SEM. *Significantly ($p < 0.05$) different from controls (C).

TABLE 2
Effect of Neonatal Estradiol Valerate Exposure on Testicular Descent

Group	Descent (days of age)
Control	23-25
PD 1	23-25
PD 1-3	27-30
PD 1-6	37-40
PD 4-6	26-26
PD 7-12	33-37
PD 13-18	30-33
PD 19-24	26-27
PD 25-30	23-25

Note. Effect of neonatal estradiol valerate exposure for various postnatal days (PD) on testicular descent ($n = 5$ for each group, except 8 for PD 1-3 group).

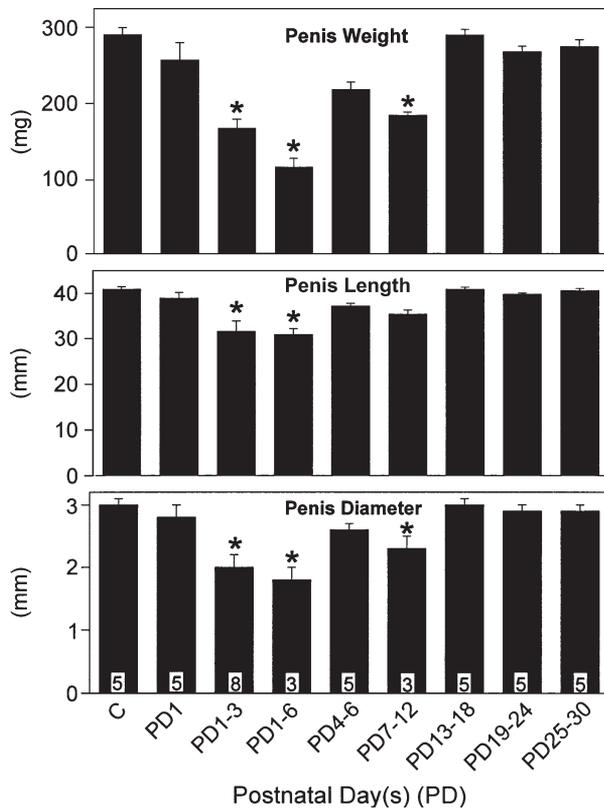


FIG. 2. Effect of neonatal estradiol valerate exposure for various postnatal days of age on the weight, length, and diameter of the penis in rats at 117–137 days of age. Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls (C).

group. Generally, the percent decrease was higher for weight than length (for example, 60 vs. 25% in the 1–6-day group).

Penile Skeletal Muscles

The weight of the bulbospongiosus muscle did not significantly ($p < 0.05$) differ from controls in groups treated after 12 days of age and in the one-day group (Fig. 3). Conversely, it was significantly lower in other groups treated prior to 12 days of age, with maximal reduction in the 1–6-day group. Significant weight reduction for the levator ani muscle was observed in the 1–6-day group only and, even within this

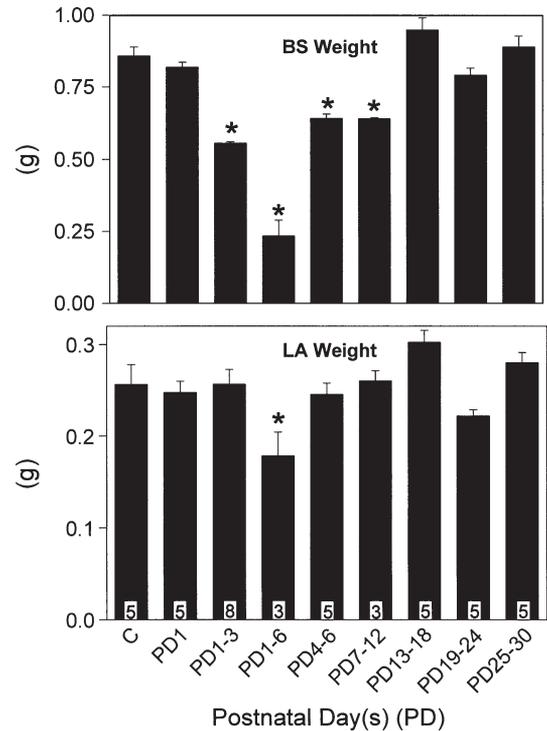


FIG. 3. Effect of neonatal estradiol valerate exposure for various postnatal days of age on the weight of the bulbospongiosus (BS) and levator ani (LA) muscles in rats at 117–137 days of age. Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls (C).

group, the percent weight reduction was much lower for the levator ani than the bulbospongiosus (30 vs. 75%).

Penile Gross Morphology, Histopathology, and Histochemistry

Gross morphology. The penis in control rats consists of a cylindrical body, a bulbous glans penis, a right angle between the body and the glans, and an os penis that extends from the distal end of the body to the tip of the glans and consists of proximal and distal parts. All these penile parts were normally developed in rats treated after 12 days of age, but exhibited various degrees of malformation in rats treated prior to 12 days of age, except those in the one-day group (Fig. 4). Generally,

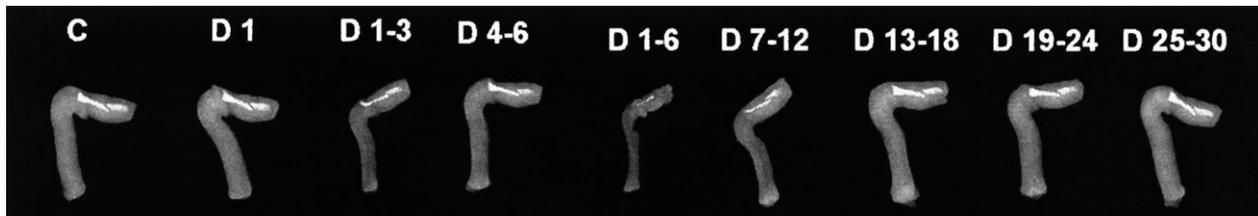
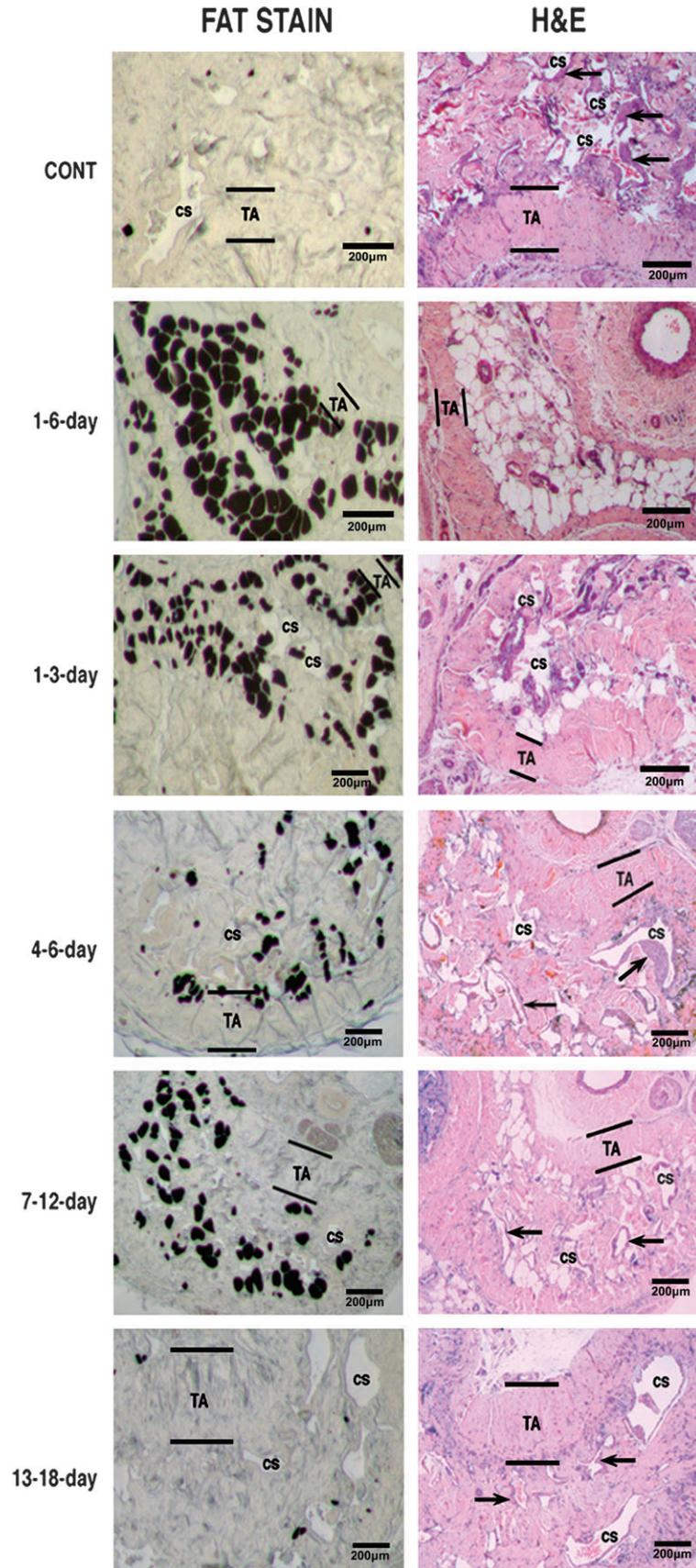


FIG. 4. Radiographs of the penis at 117–137 days of age in control (C) rats and in rats treated neonatally with estradiol valerate for various postnatal days (D) of age. Generally, note abnormal penile morphology, including mal-development and under-calcification of the os penis, in animals treated prior to 12 days of age, except in the D 1 group.



malformations included a reduction in the angle between the body and the glans and under-development and/or under-calcification of the os penis, and were most pronounced in the 1–6-day group.

Histopathology. The body of the rat penis consists of a paired corpora cavernosa that are located dorsolateral to the urethra and a corpus spongiosus that is located ventrally and surrounds the urethra. The corpora cavernosa contain endothelial-lined cavernous spaces (also called sinusoids or lacunae), smooth muscle cells, and collagen trabeculae; and are peripherally surrounded by a thick connective tissue capsule called tunica albuginea. The latter consists of an inner cellular layer, adjoining cavernous spaces, and an outer fibrous layer, which also forms a part of the outer capsule that surrounds the entire body and brings blood vessels and nerves to the penis. The corpus spongiosus is structurally similar to the corpora cavernosa, but cavernous spaces and smooth muscle cells are less developed.

Generally, all structural components of the corpora cavernosa were normally developed in rats treated after 12 days of age, but showed varying degrees of adverse effects in rats treated before 12 days of age, except in the one-day group (note, one animal in the latter group showed adverse effects similar to those observed in other animals treated prior to 12 days of age). Specifically, effects included reductions in the thickness of tunica albuginea, especially in its fibrous layer, and collagen trabeculae separating cavernous spaces, the loss of cavernous spaces and associated smooth muscle cells, and an abnormal accumulation of fat cells (Fig. 5). Comparatively, these effects were most pronounced in the 1–6-day group and the least in the 4–6-day group. Actually, fat cells appeared to have replaced the entire cavernous spaces and smooth muscle cells in the corpora cavernosa of the 1–6-day group. Unlike the corpora cavernosa, the corpus spongiosus did not show any visible evidence of loss of cavernous spaces or infiltration by fat cells, whether animals were treated prior to or after 12 days of age.

Histochemistry. Undeparaffinized sections stained with osmium tetroxide were examined to determine morphometric effects of neonatal estrogen exposure on fat cells in the corpora cavernosa penis. The mean numbers of fat cells in a unit area,

FIG. 5. Micrographs of the corpora cavernosa penis from the body of the penis at 117–137 days of age in control (CONT) rats and in rats treated with estradiol valerate for various postnatal days of age. Depending upon the timing and/or length of exposure, note varying degrees of reduction in the thickness of tunica albuginea (TA), accumulation of fat cells (stained black), and loss of cavernous spaces (cs) and smooth muscle cells (arrows) in rats treated prior to 12 days of age, with fat cells virtually replacing all cavernous spaces and smooth muscle cells in the 1–6-day group. Note the lack of these effects in the 13–18-day group. Micrographs from the 19–24- and 25–30-day groups are not included because they were similar to those of the 13–18-day group and controls. Fat stain, undeparaffinized sections stained en block with osmium tetroxide; H&E, hematoxylin and eosin.

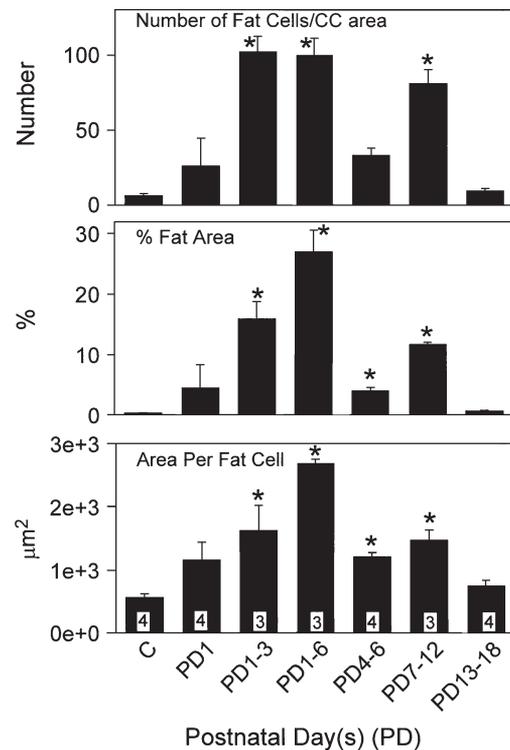


FIG. 6. Effect of neonatal estradiol valerate exposure for various postnatal days of age on numbers of fat cells in a unit area, percent area occupied by fat cells in a unit area, and area per fat cell in the corpora cavernosa (CC) in rats at 117–137 days of age. Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls (C).

the mean percent area occupied by fat cells in a unit area, and the mean area per fat cell were, respectively, 6.3, 0.368%, and 566 μm^2 in controls. None of these parameters was significantly ($p < 0.05$) different from controls in rats treated after 12 days of age and in the one-day group (Fig. 6), but all of them were increased in other groups treated prior to 12 days of age, with increases approaching 15-, 50- and 5-fold, respectively, in the 1–3- and/or 1–6-day groups.

Plasma and Intratesticular Testosterone

The mean plasma testosterone concentration in adult rats treated neonatally with EV for various lengths of time did not significantly ($p < 0.05$) differ between controls (1.09 ng/ml) and the treated groups, except in the 1–6- and 4–6-day groups where it was significantly lower ($p < 0.05$). The mean intratesticular testosterone concentration was higher, although not significantly ($p < 0.05$), in rats treated after seven days of age. Conversely, in rats treated before seven days of age, it was similar between controls (44.3 ng/mg) and the 1-, 1–3-, and 1–6-day groups, but significantly lower in the 4–6-day group (Fig. 7).

The mean intratesticular testosterone concentration in control rats at 5 and 8 days of age was 160 and 98 ng/mg, respectively;

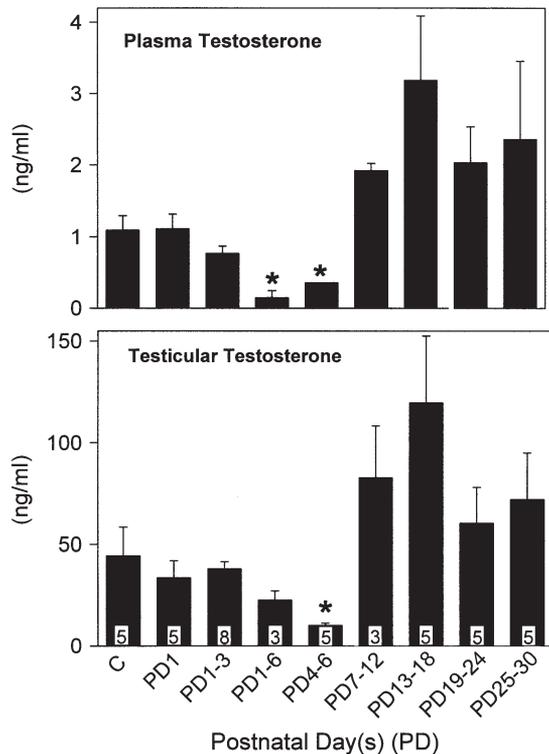


FIG. 7. Effect of neonatal estradiol valerate exposure for various postnatal days of age on plasma and intratesticular testosterone concentration in rats at 117–137 days of age. Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls (C).

however, it was reduced to 9 ng/mg at postnatal day 12 and to a nearly undetectable level at postnatal day 17 (Fig. 8). Conversely, EV exposure for 1–3 postnatal days reduced intratesticular testosterone concentration by more than 90% at 5 and 8 days of age.

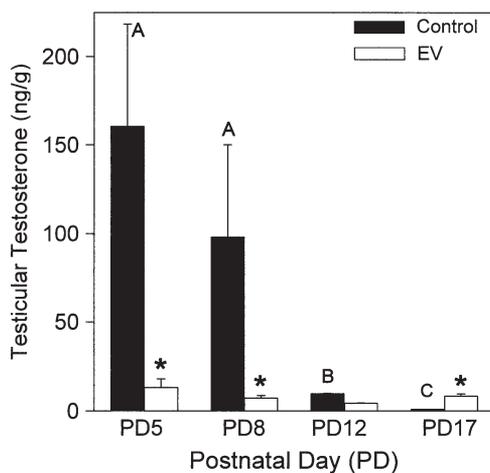


FIG. 8. Effect of neonatal estradiol valerate exposure for postnatal days 1–3 on intratesticular testosterone concentration in rats at postnatal day 5, 8, 12, and 17 ($n = 2$ for each of the control and treated groups). Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls.

Daily Sperm Production, Epididymal Sperm Numbers, and Sperm Morphology

These parameters were compared between controls and the 1–3- and 13–18-day groups. Neither the number of homogenization-resistant sperm per testis, nor the daily sperm production per gram of testicular parenchyma, and nor the number of sperm in the head and body of the epididymis or the tail of the epididymis in the 1–3-day group was significantly ($p < 0.05$) different from that of controls or the 13–18-day group (Fig. 9). The mean percentage of normal sperm ranged from 92–96% in both treatment groups and was not significantly different from that of controls. Of various sperm abnormalities, abnormal heads (1–3%) and detached heads (1–4%) were most frequently encountered in both the control and treated animals.

ER α mRNA expression

This parameter was compared between controls and the 1–3- and 13–18-day groups. The band intensity, as well as the mean signal intensity, for ER α mRNA in the body of the penis was similar between controls and the 13–18-day group, but was significantly ($p < 0.05$) higher in the 1–3-day group (Fig. 10).

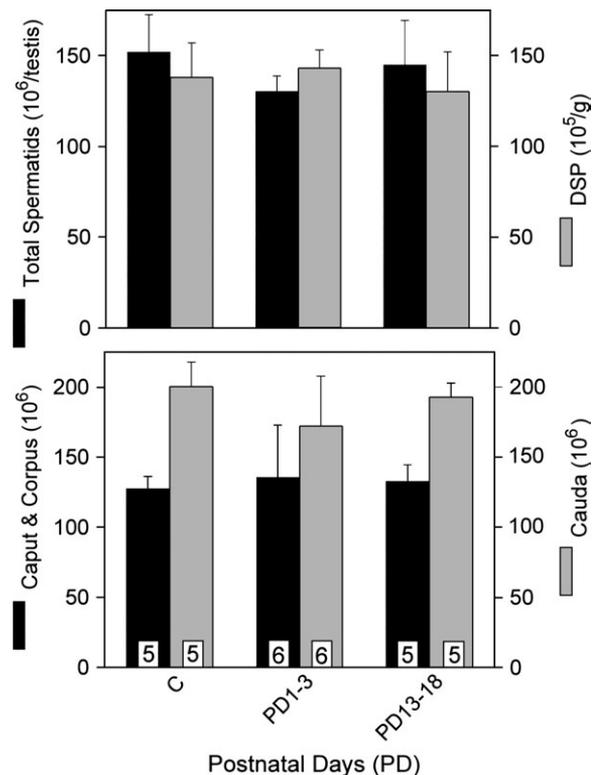


FIG. 9. Effect of neonatal estradiol valerate exposure for postnatal days 1–3 (the group that had abnormal penis) and 13–18 (the group that had normal penis) on numbers of spermatids/sperm per testis and daily sperm production (DSP) per gram of testis (upper panel) and numbers of sperm in the head and body and the tail of the epididymis (lower panel) in rats at 117–137 days of age. Data are expressed as mean \pm SEM. *Significantly ($p < 0.05$) different from controls.

DISCUSSION

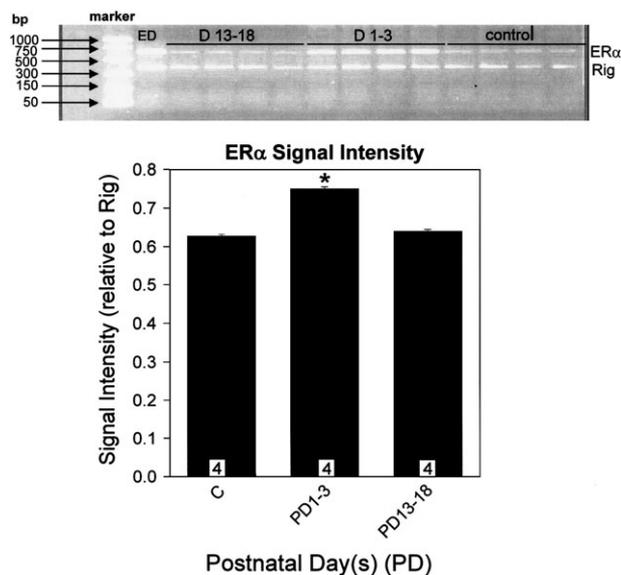


FIG. 10. Effect of neonatal estradiol valerate exposure for postnatal days 1–3 (the group that had abnormal penis) and 13–18 (the group that had normal penis) on expression of ER α mRNA in the body of the penis in rats at 117–137 days of age. Histogram (lower panel) shows ER α mRNA signal intensity relative to *Rig* (house keeping gene). Efferent ductules (ED) were included as a positive control.

Fertility

While four of five females mated with males in the control group and three of five females each mated with males in the 1-, 13–18-, 19–24-, and 25–30-day groups had pups, copulatory plugs, and sperm in the vaginal washings, none of the females mated with males in the other groups had pups, except one of four females in the 4–6-day group (Table 3).

TABLE 3
Effect of Neonatal Estradiol Valerate (EV) Exposure to Male Rats for Various Postnatal Days (PD) on Fertility at 102–115 Days of Age

Group	Pregnant/mated	Pups/litter	Implantations	Corpora lutea
Control	4/5	15, 11, 9, 15	15, 11, 10, 15	16, 13, 11, 17
PD 1	3/5	15, 15, 16	15, 15, 18	17, 15, 19
PD 1–3	0/3	N/A	N/A	N/A
PD 4–6	1/4	3	3	4
PD 1–6	0/3	N/A	N/A	N/A
PD 7–12	0/3	N/A	N/A	N/A
PD 13–18	3/5	14, 14, 16	14, 15, 16	15, 15, 20
PD 19–24	3/5	19, 15, 18	19, 15, 19	20, 15, 20
PD 25–30	3/5	13, 15, 14	13, 15, 14	15, 15, 15

Note. Individual data are presented; cohabitation with adult females (1:1) for 12 days. N/A, not applicable.

In the present study, neonatal male rat pups were treated with EV for postnatal day(s) 1, 1–3, 4–6, 1–6, 7–12, 13–18, 19–24, and 25–30 to determine if there is a critical period(s) during which estrogen exposure results in penile abnormalities and loss of fertility at adulthood. Results of the study provided clear evidence for the first time that neonatal estrogen exposure before 12 days of age, as short as 1–3 days postnatal, resulted in permanent penile abnormalities and loss of fertility in 100% of the treated rats.

Similarities in penile abnormalities reported in the present study with those previously described from our laboratory, as a result of neonatal exposure to different doses of DES or EV (Goyal *et al.*, 2005), clearly outline penis as a target organ for neonatal estrogen exposure and reveal that development of penile abnormalities is dose-dependent and requires a critical window of exposure. Although our studies are the first to report estrogen-induced histopathological changes in the penis, and associated loss of fertility in rats; laboratory animals exposed neonatally to estrogen developed smaller penis (McLachlan *et al.*, 1975; Newbold, 2004; Zadina *et al.*, 1979); small phalluses in alligators were linked to aberrant exposure to environmental estrogenic contaminants (Guillette *et al.*, 1994, 1996); and male offspring of women exposed to DES during pregnancy had smaller penis (Gill *et al.*, 1979; Shawn, 2000).

Generally, the weight reductions observed in the testis, head and body of the epididymis, tail of the epididymis, and/or seminal vesicle in rats treated with EV prior to 12 days of age, with maximal reduction in the seminal vesicle and minimal reduction in the testis, were in agreement with previous data from our laboratory (Goyal *et al.*, 2003, 2005), as well as from other laboratories (Atanassova *et al.*, 2000; Putz *et al.*, 2001; Sharpe *et al.*, 1998). In addition, similarities in percent decrease in the weight of the caudal epididymal fat pad and seminal vesicle corroborate results of our previous study (Goyal *et al.*, 2005), in which we showed for the first time that the caudal epididymal fat pad, in terms of estrogen sensitivity, is at equality with the seminal vesicle, which is one of the most known estrogen-sensitive male reproductive organs (Goyal *et al.*, 2001; Putz *et al.*, 2001; Robaire *et al.*, 1987). Although we did not determine whether fat pads in other areas of the body responded similarly, parametrial fat pads decreased in female mice treated with 17 β -estradiol or genistein (Naaz *et al.*, 2003).

Consistent with effects of neonatal estrogen exposure on penile measurements, weights of the penile skeletal muscles were also unaltered in rats treated after 12 days of age, but exhibited a differential response in animals treated prior to 12 days of age; with significant reductions present in the bulbospongiosus muscle in all treated groups, except the 1-day group, and significant reductions present in the levator ani muscle in the 1–6-day group only. These findings, in concert with similar differential effects exhibited by these muscles in

response to different doses of neonatal EV or DES exposure (Goyal *et al.*, 2005), clearly suggest that the bulbospongiosus muscle is much more sensitive to adverse effects of estrogen than the levator ani muscle. The mechanism of this differential response is unclear, but may, in part, be attributed to differences in androgen action since both muscles are androgen-dependent (Breedlove and Arnold, 1983; Mansouri *et al.*, 2003).

The most notable findings of this study, as well as those of our previous studies (Goyal *et al.*, 2004a,b, 2005), included accumulation of fat cells and loss of cavernous spaces and smooth muscles in the corpora cavernosa, but not in the corpus spongiosus, implying specificity of estrogenic effects within the penis. To our knowledge, similar histopathological effects have not been reported in the rat or any other species except the rabbit, where bisphenol A (Moon *et al.*, 2001) or tetrachlorodibenzodioxin (Moon *et al.*, 2004) treatment at puberty resulted in subtunical deposition of fat cells, decreased cavernous spaces, and increased smooth muscle cells in the corpora cavernosa. Differences between our studies and those of the latter authors may be attributed to differences in the species, estrogenic compound, and/or the time of treatment. Nevertheless, these data pointed to cavernous spaces and smooth muscles in the corpora cavernosa as the target tissues for estrogen action. Additionally, induction of the above histopathological effects in rats treated prior to 12 days of age, and the lack thereof in rats treated after 12 days of age, established postnatal days 1–12 as the period when penis was sensitive to estrogen exposure. Additional observations that the magnitude of these effects (for example, increase in the number, size, and percent area of fat cells) was higher in the 1–3-day group than in the 4–6- and 7–12-day groups determined 1–3 postnatal days as the most sensitive period. Although we do not know of any similar study that sought to determine a critical period for estrogen action in the developing penis, experiments involving castration at birth, with or without testosterone supplementation at various perinatal periods, also identified postnatal days 1–7, especially the first few days, when the rat penis was most sensitive to androgen deprivation (Beach *et al.*, 1969).

An important question that needs to be answered is: “Why is the rat penis prone to adverse effects of estrogen exposure prior to 12 days of age, especially from 1–3 days of age?” Although the present study was not designed to address this question, observations of other studies that both cavernous spaces and smooth muscle cells are absent in the corpora cavernosa at birth and start to differentiate from stromal cells at 5–7 postnatal days in the rat (Murakami, 1986, 1987) suggest that the structural changes that we observed in the penis may have resulted from estrogen-induced alterations in stromal cell differentiation. The normal development of the penis and fertility in adult rats treated with estrogen from postnatal days 13–18 or 19–24 or 25–30, when cavernous spaces and smooth muscle cells are already differentiated (Goyal *et al.*, 2004b; Murakami, 1987), provide support to the above suggestion.

Hence, we hypothesize that estrogen exposure during a critical developmental period (1–12 postnatal days, especially 1–3 postnatal days) induces permanent alterations in differentiation and proliferation of stromal cells, which may, in turn, be redirected to develop into adipocytes at the expense of endothelial and/or smooth muscle cells in the corpora cavernosa. It is worth noting that the rat penis during this estrogen-sensitive period is similar developmentally to the human penis in the first and second trimesters of pregnancy (George and Wilson, 1994; Klonisch *et al.*, 2004; Williams-Ashman and Reddi, 1991) when mothers of more than two million men in the world (1940–1970) received DES for preventing miscarriage (Shawn, 2000).

The extent to which observed effects of estrogen on penile organization may result from direct estrogen action via the ER, versus indirect effects via estrogen-induced alterations in testosterone and associated changes in androgen action, remains unclear. Support for a direct effect comes from observations that both ER α and β are present in stromal cells in one-day-old rat penis (Jesmin *et al.*, 2002), that estrogens inhibit proliferation of smooth muscles of injured blood vessels (Goyal and Oparil, 2001), and that the estrogen metabolite, 2-methoxyestradiol, inhibits angiogenesis (Fotsis *et al.*, 1994). Importantly, the present observations of up-regulation of ER α in the 1–3-day group (the group in which rats had abnormal penis), but not in the 13–18-day group (the group in which rats had normal penis), as well as our previous observations of enhanced expression of ER α in stromal cells of the corpus cavernosum at 18 days of age in rats treated neonatally with DES (Goyal *et al.*, 2004b), lend credence to the direct estrogen effect hypothesis. Neonatal estrogen exposure was reported to up-regulate ER α expression in the mouse uterus (Yamashita *et al.*, 1990), murine male reproductive tract (Sato *et al.*, 1994), and rat prostate (Prins and Birch, 1997; Prins *et al.*, 2001). ER α knockout mice exhibited resistance to the developmental abnormalities of neonatal DES exposure in the female genital tract (Couse *et al.*, 2001).

Alternatively, androgen receptors (AR) are present throughout the body of the rat penis (Goyal *et al.*, 2004b); AR concentration reaches a peak level at or prior to puberty (Rajfer *et al.*, 1980; Takane *et al.*, 1990); neonatal estrogen exposure reduces AR expression in male reproductive organs (McKinnell *et al.*, 2001; Prins and Birch, 1995; Williams *et al.*, 2001; Woodham *et al.*, 2003) and lowers plasma testosterone (Atanassova *et al.*, 2000; Sharpe *et al.*, 1998); and co-administration of testosterone with DES prevents most of the histopathological abnormalities affecting the male reproductive tract in rats (Rivas *et al.*, 2003). Collectively, these observations raise the possibility of lower testosterone and/or decreased AR (or AR activation) as factors contributing indirectly to development of estrogen-induced penile abnormalities. Although, in a previous study, we did not find differences in AR expression in the penis following neonatal estrogen exposure, plasma testosterone was decreased (Goyal

et al., 2004b), implying lower androgen action. Furthermore, results of the present study that EV exposure for 1–3 postnatal days reduced intratesticular testosterone by almost 90% at postnatal day 5 or 8, the developmental period when stromal cells start differentiation into cavernous spaces and smooth muscles in the rat penis (Murakami, 1986, 1987), provide credence to the indirect effect hypothesis, via lower androgen action, in altering stromal cell differentiation. It is noteworthy that androgens promoted smooth muscle differentiation and inhibited adipocyte differentiation from stromal progenitor cells (Bhasin *et al.*, 2003); and castration induced subcutaneous fat deposition and loss of smooth muscles in the corpora cavernosa of the rabbit penis (Traish *et al.*, 2005).

Finally, the absence of copulatory plugs and vaginal sperm in females mated with males treated with EV prior to 12 days of age suggests a deficit in intromission and/or ejaculation, although measurements of intracavernosal blood pressure in response to pelvic nerve stimulation are warranted for confirmation. Considering the partial attachment of preputial sheath and the loss of cavernous spaces and smooth muscle cells, histological structures responsible for erection (Benson, 1994), it is highly unlikely that these animals would be able to intromit or attain erection sufficient enough for completing a successful copulatory act, including ejaculation. Similarities in numbers of sperm per testis, daily sperm production, sperm reserve in the tail of the epididymis, and sperm morphology between controls and the 1–3-day group (the group in which all animals had abnormal penis) and the 13–18-day group (the group in which all animals had normal penis), rule out deficits in sperm function as a probable cause of loss of fertility in animals treated with estrogen for 1–3 days of age; although this cause, in the absence of intromission or ejaculation, is immaterial.

In conclusion, the present study provides evidence for the first time that exposure to EV prior to 12 days of age, as short as 1–3 postnatal days, results in permanent penile deformities and loss of fertility.

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